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Blocking angiotensin II ameliorates proteinuria and glomerular lesions in progressive mesangioproliferative glomerulonephritis

Takamichi Nakamura, Jun-ei Obata, Hideaki Kimura, Shinichi Ohno, Yoji Yoshida, Hiroshi Kawachi, and Fujio Shimizu

Division of Blood Transfusion, Department of Internal Medicine, Department of Anatomy, Department of Pathology, Yamanashi Medical University, Yamanashi, and Department of Cell Biology, Institute of Nephrology, Niigata University School of Medicine, Niigata, Japan

Blocking angiotensin II ameliorates proteinuria and glomerular lesions in progressive mesangioproliferative glomerulonephritis.

Background. The renin-angiotensin system is thought to be involved in the progression of glomerulonephritis (GN) into end-stage renal failure (ESRF) because of the observed renoprotective effects of angiotensin-converting enzyme inhibitors (ACEIs). However, ACEIs have pharmacological effects other than ACE inhibition that may help lower blood pressure and preserve glomerular structure. We previously reported a new animal model of progressive glomerulosclerosis induced by a single intravenous injection of an anti-Thy-1 monoclonal antibody, MoAb 1-22-3, in uninephrectomized rats. Using this new model of progressive GN, we examined the hypothesis that ACEIs prevent the progression to ESRF by modulating the effects of angiotensin II (Ang II) on the production of transforming growth factor-β (TGF-β) and extracellular matrix components.

Methods. We studied the effect of an ACEI (cilazapril) and an Ang II type 1 receptor antagonist (candesartan) on the clinical features and morphological lesions in the rat model previously reported. After 10 weeks of treatment with equihypotensive doses of cilazapril, cilazapril plus Hoe 140 (a bradykinin receptor B2 antagonist), candesartan, and hydralazine, we examined systolic blood pressure, urinary protein excretion, creatinine clearance, the glomerulosclerosis index, and the tubulointerstitial lesion index. We performed a semiquantitative evaluation of glomerular immunostaining for TGF-β and collagen types I and III by immunofluorescence study and of these cortical mRNA levels by Northern blot analysis.

Results. Untreated rats developed massive proteinuria, renal dysfunction, and severe glomerular and tubulointerstitial injury, whereas uninephrectomized control rats did not. There was a significant increase in the levels of glomerular protein and cortical mRNA for TGF-β and collagen types I and III in untreated rats. Cilazapril and candesartan prevented massive proteinuria, increased creatinine clearance, and ameliorated glomerular and tubulointerstitial injury. These drugs also reduced levels of glomerular protein and cortical mRNA for TGF-β and collagen types I and III. Hoe 140 failed to blunt the renoprotective effect of cilazapril. Hydralazine did not exhibit a renoprotective effect.

Conclusion. These results indicate that ACEIs prevent the progression to ESRF by modulating the effects of Ang II via Ang II type 1 receptor on the production of TGF-β and collagen types I and III, as well as on intrarenal hemodynamics, but not by either increasing bradykinin activity or reducing blood pressure in this rat model of mesangial proliferative GN.

Key words: renin-angiotensin system, TGF-β, ACE inhibitors, bradykinin, progressive renal disease.

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Angiotensin-converting enzyme inhibitors (ACEIs) effectively reduce or even reverse the development of sclerotic lesions in animal models of progressive glomerular sclerosis [1–4]. ACEIs decrease angiotensin II (Ang II) generation [5] and increase renal kallikrein activity [6], plasma and renal kinin levels [7], and urinary kinin excretion [8]. Bradykinin selectively dilates the efferent arterioles [9] and enhances the formation of nitric oxide and prostacylin [10], to which the renoprotective effect of ACEI may be attributed. In passive Heymann nephritis, ACEIs reduce proteinuria through their action on the kallikrein-kinin system rather than on the renin-angiotensin system (RAS) [11–13]. On the other hand, both Ang II type 1 receptor (AT1R) antagonists and ACEIs reduce proteinuria and glomerulosclerosis in remnant kidney [14, 15] and hypertensive glomerulosclerosis models [16], suggesting that RAS plays an important role in the occurrence of proteinuria and glomerulosclerosis in these models. However, the effect of the AT1R antagonist may not be entirely due to the blockade of AT1R. Angiotensin receptors comprise two major subtypes, AT1 and AT2 [17]. Because the AT1R antagonist increases plasma renin and angiotensins, increased angio-

tensins may act on AT2R, which could have an antiproliferative effect [18] and, consequently, contribute to the therapeutic effect of AT1R antagonist.

Expansion of the mesangial area, caused by proliferation or hypertrophy of mesangial cells accompanied by an increase in the extracellular matrix, is a major pathological feature of glomerular diseases that often progress into end-stage renal failure (ESRF) [19]. Accumulating evidence suggests that transforming growth factor-β (TGF-β) plays a pivotal role in the progression of renal disease [20, 21]. Yamamoto et al reported that in a rat with progressive glomerulonephritis (GN) induced by two consecutive injections of anti-Thy-1 antibody, TGF-β levels were elevated by infiltrating platelets and macrophages in the initial phase and were kept high by activated resident glomerular cells in the late phase [22]. TGF-β autoinduces its own production, which greatly amplifies its biological actions [23]. Ang II also increases the production of TGF-β in cultured mesangial cells [24]. Glomerular hypertension increases the protein and mRNA levels of TGF-β in the glomerulus [25]. In contrast, nitric oxide suppresses TGF-β increased by thromboxane in cultured mesangial cells [26]. Ruiz-Ortega et al demonstrated that an ACEI attenuated the development of massive proteinuria, ameliorated the morphological lesions, and decreased the gene expression of TGF-β and matrix proteins in rat immune-complex GN that morphologically resembles human mesangiocapillary GN, although it remains unclear whether the renoprotective effect of ACEI is due to the reduction of Ang II generation [27].

Recently, we reported a new animal model of progressive mesangioproliferative GN with marked proteinuria, induced by a single intravenous injection of anti-Thy-1 monoclonal antibody, MoAb 1-22-3, to uninephrectomized rats [28]. Immunofluorescence (IF) studies revealed an increase in glomerular expression of TGF-β in this model. Furthermore, we have found that in this model, pirfenidone, an antifibrotic agent, decreased the glomerular deposition of TGF-β and protected the kidney from the progression to ESRF [29]. These results, combined with the findings mentioned earlier here [14–16, 27], raised the possibility that in this model, RAS blockade could prevent the progression to ESRF by reducing the production of TGF-β and matrix proteins. Therefore, using this model, we investigated whether an ACEI and an AT1R antagonist show renoprotective effects, whether a bradykinin type 2 receptor antagonist modifies the effect of an ACEI on the course of the disease, and whether these agents change the protein and mRNA levels of TGF-β and collagen types I and III.

METHODS

Animals and protocol

Six-week-old male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) were housed in individual cages at a constant room temperature (23 to 25°C) with a 12-hour light/dark cycle; the animals were allowed to eat standard feed (24% protein) and drink control water (distilled water) *ad libitum.* Progressive mesangioproliferative GN was induced, with minor modifications, according to a previously described protocol [28, 29]. Briefly, rats were uninephrectomized and, two weeks afterward, were injected intravenously with 0.5 mg of anti-Thy-1 monoclonal antibody (MoAb 1-22-3). On the day when anti-Thy-1 monoclonal antibody was injected, animals were randomly assigned to five groups and were treated as follows. The untreated group ($N = 6$) comprised animals with nephritis and no treatment. The cilazapril-treated group ($N = 6$) comprised animals treated with the ACEI, cilazapril (as powdered hydrochloride; Eizai, Tokyo, Japan) in the drinking water. The concentration of cilazapril was 10 mg/liter to allow administration of 1 mg/kg body wt/day of cilazapril, because the animals drank approximately 25 ml of water a day.

The cilazapril plus Hoe 140-treated group ($N = 6$) comprised animals treated with cilazapril and the bradykinin B2-receptor antagonist, Hoe 140 (Hoechst AG, Frankfurt, Germany), which was administered chronically by subcutaneous infusion via osmotic minipumps (Mini-osmotic pump model 2002; Alza Corp., Palo Alto, CA, USA). The minipump filled with Hoe 140 was subcutaneously implanted in the abdominal wall of the rats under ether anesthesia, and a dose of 500 μg/kg body wt was injected daily [30]. The minipumps were replaced every two weeks.

The candesartan-treated group ($N = 6$) comprised animals injected intraperitoneally with 1 ml/kg body wt/day of the AT1R antagonist candesartan (Takeda Chemical Industries, Osaka, Japan), which was solubilized in phosphate-buffered saline (PBS; pH 7.2) at a concentration of 0.5 mg/ml to administer at 0.5 mg/kg body wt/day as previously described [31].

The hydralazine-treated group ($N = 6$) comprised animals treated with hydralazine (as powdered hydrochloride; Ciba-Geigy Japan, Takarazuka, Japan) in the drinking water. The concentration of hydralazine was 50 mg/liter to allow administration of 5 mg/kg body wt/day.

The uninephrectomized control group ($N = 6$) comprised animals injected with physiological saline instead of antibody.

To examine the effects of the repeated implantations of the minipumps and daily intraperitoneal injections on the progression of the disease, we performed the following experiment. After uninephrectomized animals were injected with antibody, they were randomly assigned to three groups and treated as follows.

The minipump-implanted group ($N = 6$) comprised rats with a subcutaneously implanted minipump filled with PBS. The minipumps were replaced every two weeks.

The PBS-injected group ($N = 6$) comprised animals injected intraperitoneally with 1 ml/kg body wt/day of PBS.

The untreated group ($N = 6$) comprised animals with nephritis and no treatment.

The uninephrectomized control group ($N = 6$) comprised animals that were injected with physiological saline instead of antibody.

Effect of treatment on the binding of antibody in the kidney

To examine the effect of antihypertensive agents on the binding of anti-Thy-1 monoclonal antibody to the glomeruli, we started the administration of candesartan or cilazapril (three rats in each group) one day before the injection of 500 μg of ^{125}I-labeled MoAb 1-22-3. These animals were killed 30 minutes after injection, and radioactivity in the kidney was measured as previously described [32], as this monoclonal antibody binds only to the mesangial cells in the kidney [33]. MoAb 1-22-3 was labeled with ^{125}I by the chloramine-T method [34]. Labeled preparations in which 98% of all isotopes were protein bound were used. Specific activity was 45,673 ct/min per μg of ^{125}I-labeled MoAb. Radioactivity was measured in an autogamma scintillation photometer (model 5260; Packard, Sterling, VA, USA).

Proteinuria and renal function

Urine was collected every two weeks from rats housed for 24 hours in metabolic cages with access to water only. The amount of urinary protein (U_{Prot}) excreted was measured by the Pyrogallol Red method. At the end of the study period, serum levels of creatinine were measured according to the standard method. Creatinine clearance (C_{Cr}) was calculated from a urine sample taken 24 hours before the animal was sacrificed.

Blood pressure measurement

Systolic blood pressure (SBP) was measured in conscious, restrained rats using a tail-cuff sphygmomanometer every two weeks after the induction of nephritis. The SBP for each rat was calculated as the average of three separate measurements at each session.

Kidney tissue processing

At the time of sacrifice, animals were anesthetized with ether. The kidneys were perfused *in vivo* via the abdominal aorta with 50 ml of physiological saline at 4°C, removed immediately, and processed further for histologic studies and RNA extraction.

Renal histologic studies

Tissue was fixed in buffered formalin and was embedded in paraffin. Formalin-fixed tissue was sectioned (3 to 4 μm) and stained with periodic acid-Schiff. Glomerular sclerosis was assessed as follows using a semiquantitative score previously described [34, 35]: grade 0, normal appearance; grade I, involvement of up to 25% of the glomerulus; grade II, involvement of 25 to 50% of the glomerulus; grade III, involvement of 50 to 75% of the glomerulus; grade IV, involvement of 75 to 100% of the glomerulus. A glomerulosclerosis index (GSI) was calculated by multiplying the number of glomeruli with a sclerosis score of I by one, the number with a score of II by two, III by three, and IV by four. These values were summed and divided by the number of glomeruli assessed, including those with a sclerosis score of zero. A minimum of 150 glomeruli were examined from each specimen. The tubulointerstitial lesion index (TILI; matrix deposition and mononuclear cell infiltration) was assessed in 20 randomly selected cortical areas per sample and was observed at a magnification of ×250 using the following scale: 0 = normal, 1 = lesions involving less than 10% of the cortical area, 2 = involving 10 to 30%, 3 = involving 30 to 50%, and 4 = involving more than 50%.

For the immunostaining for TGF-β and collagen types I and III, kidney tissue was embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek USA, Inc., Torrance, CA, USA), snap-frozen in *N*-hexane cooled at −70°C, and stored at −70°C until the study. Frozen tissues were sectioned at 2 to 3 μm thickness using a cryostat. For the immunostaining for TGF-β, sections were pretreated with acid urea, as described by Yoshioka et al [36], and were incubated with rabbit anti-TGF-β antibody (a gift from Dr. Muramatsu, Research Center, Mitsubishi Kasei, Yokohama, Japan; produced by the method described by Flanders et al [37]) for 30 minutes at room temperature. For the immunostaining for type I and III collagens, sections were washed in PBS and incubated with rabbit antibodies against type I and III collagens (Chemicon International Inc., Temecula, CA, USA) for 30 minutes, as previously described [38]. After being washed in PBS, the sections were incubated with fluorescein isothiocyanate-labeled antirabbit immunoglobulins (Dako, Glostrup Denmark) for 30 minutes. The sections were washed in PBS, mounted in buffered glycerol, and examined with an Olympus fluorescent microscope.

For each kidney, approximately 10 to 30 glomeruli were assessed according to the degree of IF staining for TGF-β and collagen type I and type III. Glomerular expression was graded semiquantitatively according to the following procedure described by Kimura et al [39]: 0 = no expression; 1 = mild expression (less than 30% of a glomerular area); 2 = moderate expression (30 to 60% of a glomerular area); 3 = marked expression (more than 60% of a glomerular area). An expression score was calculated by multiplying the number of glomeruli with a expression score of 1 by one, the number with a score of 2 by two, and 3 by three. These values were summed and divided by the number of glomeruli assessed, including those with an expression score of zero.

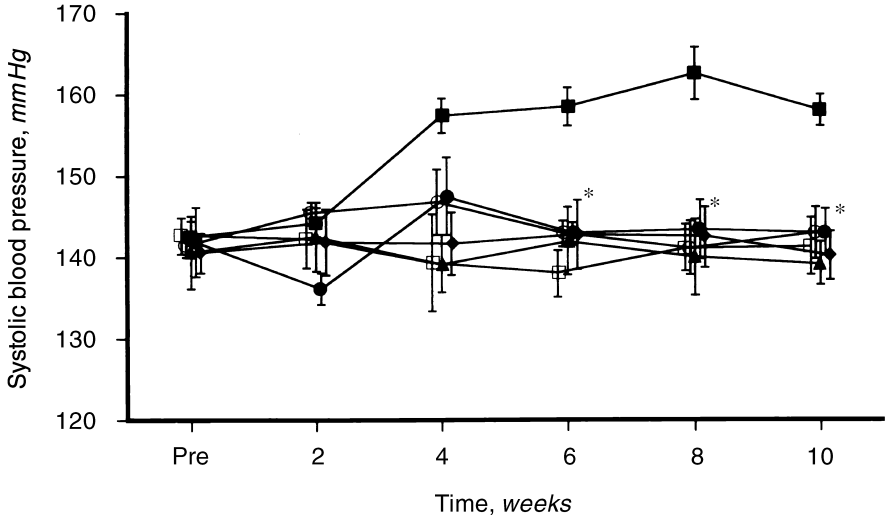

Fig. 1. Time course of systolic blood pressure. Symbols are: (■) untreated glomerulonephritis (GN) rats; (◆) hydralazine-treated GN rats; (▲) cilazapril-treated GN rats; (●) cilazapril plus Hoe 140-treated GN rats; (□) candesartan-treated GN rats; (○) nephrectomized control rats. $*P < 0.05$ vs. untreated GN rats.

Extraction of cortical total RNA and Northern blot hybridization

In three separate experiments, kidney tissue from two animals was divided into the cortex and medulla portions, and cortical tissue was used for RNA isolation. Total RNA from cortical tissue was extracted by the single-step method of Chomczynski and Sacchi [40]. Ten micrograms of total RNA were separated by electrophoresis on 1.0% agarose formaldehyde gels, transferred to a nylon filter (Hybond N), cross-linked by ultraviolet illumination, and hybridized with [^{32}P]dCTP random-oligolabeled cDNA probes for rat TGF-β (a generous gift from Dr. T. Nakamura) [41], rat α 1 (I) collagen (a generous gift from Dr. C. Genovese) [42], rat α 1 (III) collagen (a generous gift from Dr. Y. Yamada) [43], and GAPDH (a generous gift from Dr. P. Fort) [44]. cDNA probes were radiolabeled by random oligolabeling with DNA polymerase I (Takara Co. Ltd., Kyoto, Japan) in the presence of [^{32}P]dCTP (3000 Ci/mmol). For hybridization, the radiolabeled probe (total count, 10^6 cpm) and 100 μl (50 mg/ml) of salmon sperm DNA were added to this solution for 24 hours at 42°C. The membranes were washed three times. The radioautographs were quantitated by laser densitometry (Fujitsu Co. Ltd., Kyoto, Japan).

Statistical analysis

All values are expressed as the mean ± standard error of the mean. For blood pressure and the urinary protein excretion (U_{Prot}), statistical significance was evaluated using analysis of variance with modified *t*-tests. The semiquantitative evaluation of the immunostaining was compared between groups with nonparametric analysis based on the Kruskal–Wallis test.

RESULTS

Blood pressure

In untreated animals, the SBP increased gradually starting from six weeks after the induction of nephritis, and it was significantly elevated compared to the SBP in nephrectomized controls (Fig. 1). Cilazapril, cilazapril plus Hoe 140, candesartan, and hydralazine significantly and equally prevented SBP from elevating, maintaining the SBP at the same level as that in nephrectomized control rats throughout the experiment.

At the end of the experiment, SBP was significantly elevated in animals in which a minipump was implanted subcutaneously, in those in which daily PBS was injected intraperitoneally, and in untreated nephritic animals compared with SBP in nephrectomized control animals (Table 1).

Effect of antihypertensive agents on the binding of the antibody to the kidney

The amount of total kidney-fixing antibody from control rats injected with 500 μg of MoAb 1-22-3 at 30 minutes was 7.5 ± 1.3 μg/kidney. Neither candesartan, cilazapril, nor hydralazine affected the binding of MoAb 1-22-3 to the kidney (7.8 ± 1.9, 7.8 ± 1.7, 8.4 ± 1.5 μg/kidney, respectively). This result indicates that the beneficial effects of Ang II blockade are not due to a decrease in the binding of the antibody to the kidney.

Proteinuria, creatinine clearance, and body weight

There was no difference in 24-hour U_{Prot} among the five groups before the induction of nephritis. As shown in Figure 2, U_{Prot} significantly increased one week after the induction of nephritis compared with that in nephrectomized control rats. In untreated animals, U_{Prot} increased

Table 1. Effect of manipulations on U_{Prot}, C_{Cr}, SBP, and renal pathology in nephritic rats

	U_{Prot}	C_{Cr}	SBP	GSI	TILI
PBS	130.3 ± 10.9	0.46 ± 0.14	169 ± 4	1.26 ± 0.13	2.11 ± 0.28
Minipump	135.3 ± 7.2	0.41 ± 0.14	160 ± 4	1.44 ± 0.13	1.98 ± 0.28
Untreated	133.4 ± 10.7	0.61 ± 0.08	163 ± 14	1.44 ± 0.13	2.18 ± 0.36
Non-GN	13.8 ± 1.0[a]	2.47 ± 0.32[a]	143 ± 4[a]	0.07 ± 0.01[a]	0 ± 0[a]

Abbreviations are: U_{Prot}, 24 hour urinary protein excretion; C_{Cr}, creatinine clearance; SBP, systolic blood pressure; GSI, glomerulosclerosis index; TILI, tubulointerstitial lesion index. Data are expressed as mean ± SEM.

[a] $P < 0.001$ vs. untreated

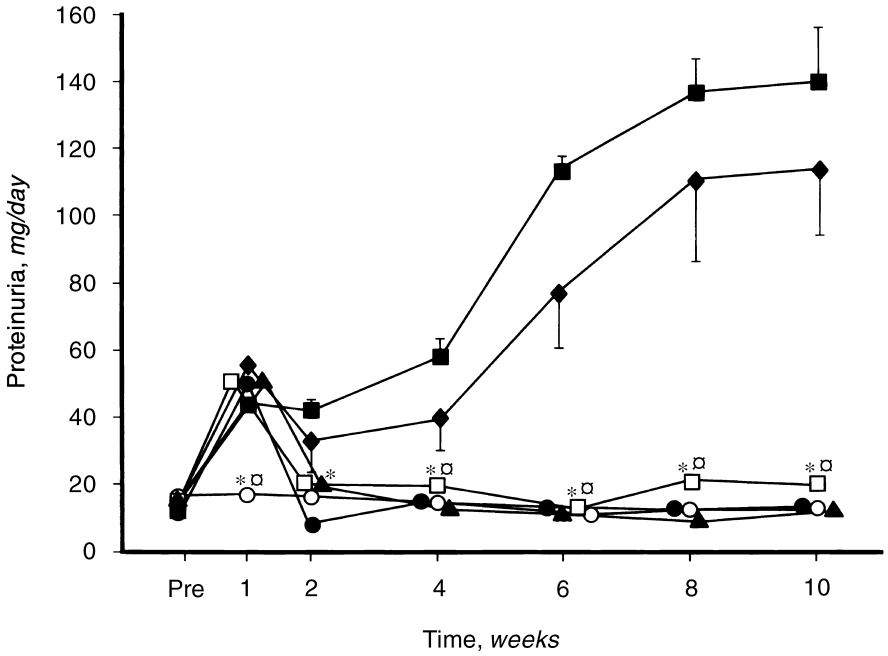

Fig. 2. Time course of urinary protein excretion. Symbols are: (■) untreated glomerulonephritis (GN) rats; (◆) hydralazine-treated GN rats; (▲) cilazapril-treated GN rats; (●) cilazapril plus Hoe 140-treated GN rats; (□) candesartan-treated GN rats; (○) nephrectomized control rats. *$P < 0.05$ vs. untreated GN rats; □$P < 0.05$ vs. hydralazine-treated GN rats.

gradually until the end of the experiment. Cilazapril, cilazapril plus Hoe 140, and candesartan decreased U_{Prot} to control levels starting from two weeks after the induction of nephritis. Hydralazine failed to prevent the progressive increase in U_{Prot}.

Creatinine clearance was significantly decreased in untreated animals (0.60 ± 0.12 ml/min/kg body wt) compared with that in nephrectomized control animals (1.94 ± 0.13 ml/min/kg body wt, $P < 0.0001$). Cilazapril, cilazapril plus Hoe 140, and candesartan significantly increased creatinine clearance to the control level (1.79 ± 0.24, 1.93 ± 0.26, and 2.16 ± 0.22 ml/min/kg body wt, respectively), whereas hydralazine significantly increased creatinine clearance (1.06 ± 0.07 ml/min/kg body wt, $P < 0.02$), but not to the control level.

All groups of animals gained body weight during the experiment (Fig. 3). However, body weight was significantly lower in untreated and hydralazine-treated GN animals compared with that in nephrectomized control rats starting from two weeks after induction of disease to the end of the study. The body weights of cilazapril-treated, cilazapril plus Hoe 140-treated, and candesartan-treated GN animals were lower than those in nephrectomized controls until six weeks after induction of the disease and then became similar. Body weight did not differ among GN animal groups until eight weeks after the induction of disease. At 10 weeks after induction of disease, body weight was lower in untreated and hydralazine-treated GN animals than in cilazapril-treated, cilazapril plus Hoe 140-treated, and candesartan-treated GN animals.

In minipump-implanted, PBS-injected, and untreated groups, U_{Prot} was increased, and creatinine clearance was decreased compared with values in nephrectomized control animals at the end of the experiment (Table 1). Body weight was significantly lower in animals in which a minipump was implanted (288 ± 7 g), in those in which PBA was injected daily intraperitoneally (286 ± 7 g), and in untreated nephritic animals (290 ± 9 g) compared with that in uninephrectomized control animals (331 ± 6 g, $P < 0.05$ vs. untreated GN rats). There were no significant differences in body weight among nephritic groups.

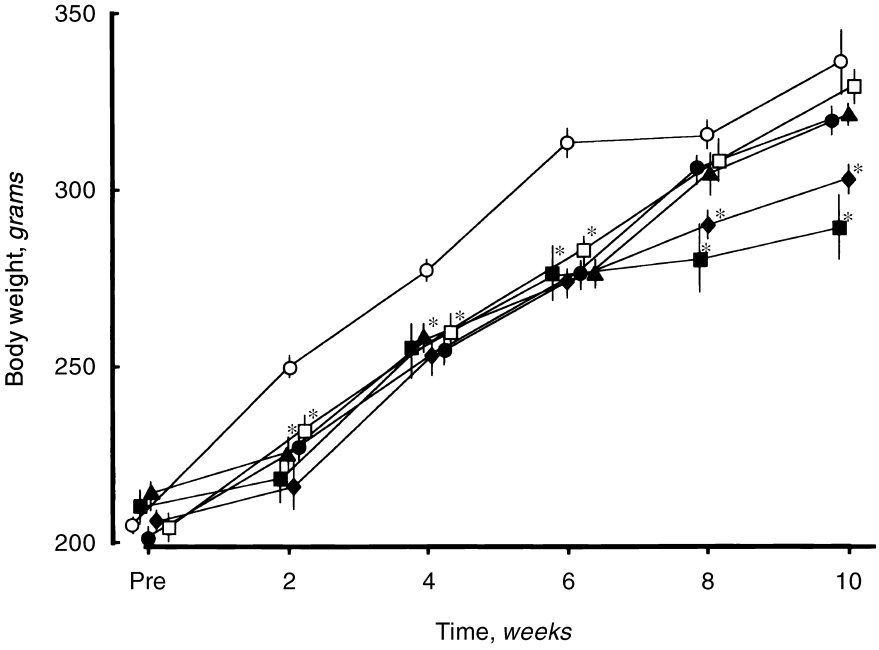

Fig. 3. Time course of body weight. Symbols are: (■) untreated glomerulonephritis (GN) rats; (◆) hydralazine-treated GN rats; (▲) cilazapril-treated GN rats; (●) cilazapril plus Hoe 140-treated GN rats; (□) candesartan-treated GN rats; (○) nephrectomized control rats. $*P < 0.05$ vs. nephrectomized control rats.

Morphology

Light microscopy. Mesangial cell proliferation and mesangial matrix expansion were observed in more than 90% of the glomeruli in untreated animals (Fig. 4). Inflammatory cell infiltration and fibrosis were also observed in the interstitium of untreated animals. Cilazapril, cilazapril plus Hoe 140, and candesartan improved the pathological changes seen in untreated animals (Fig. 4), whereas hydralazine slightly improved the pathological changes in untreated animals (Fig. 4). A semiquantitative analysis showed that the GSI and TILI in untreated animals were high compared with values in nephrectomized controls (Fig. 5). Cilazapril, cilazapril plus Hoe 140, and candesartan markedly decreased GSI, but not to the control level. Hydralazine decreased GSI and TILI slightly, but not significantly.

Neither subcutaneous minipump implantation nor daily intraperitoneal PBS injection influenced the morphological changes seen in GN animals (Fig. 6 and Table 1).

Immunohistochemistry

Representative glomeruli stained for TGF-β and collagens type I and III are shown in Figures 7, 8, and 9. Expression scores of TGF-β type I and III collagens are shown in Figure 10. TGF-β was mainly localized in the mesangial area in more than 90% of the glomeruli from the untreated rats and segmentally in the mesangial area of a few glomeruli from nephrectomized control rats. Cilazapril, cilazapril plus Hoe 140, and candesartan reduced glomerular IF staining for TGF-β to the level of nephrectomized control rats, although hydralazine did not. Type I and type III collagens were mainly localized in the mesangial area in more than 90% of glomeruli from untreated rats and segmentally in the mesangial area of a few glomeruli from nephrectomized control rats. Cilazapril, cilazapril plus Hoe 140, and candesartan markedly reduced glomerular IF staining for collagens type I and type III, but not to the control level of nephrectomized rats. Hydralazine did not change the glomerular IF staining for type I and type III collagens.

Northern blot analysis

Northern blot analysis showed that gene expression of TGF-β1 and collagens type I and III in untreated rats was increased compared with that in nephrectomized control rats (Fig. 11). Cilazapril, cilazapril plus Hoe 140, and candesartan reduced gene expression of these proteins to control levels. Hydralazine also reduced gene expression, but not to control levels.

DISCUSSION

Several studies have attempted to compare the renoprotective effects of ACEIs and AT1R antagonists [16, 45–47], and almost all have failed to distinguish any substantial differences in their effects, with some exceptions [48, 49]. ACEIs increase bradykinin concentrations, with sequential generation of nitric acid and prostaglandins [7, 10], to which the beneficial effect of ACEIs may be attributed. AT2R has been shown to exert an antiproliferative effect [18], which may contribute to the beneficial effect of AT1R antagonists. Because of the possibility that bradykinin and pathways mediated by

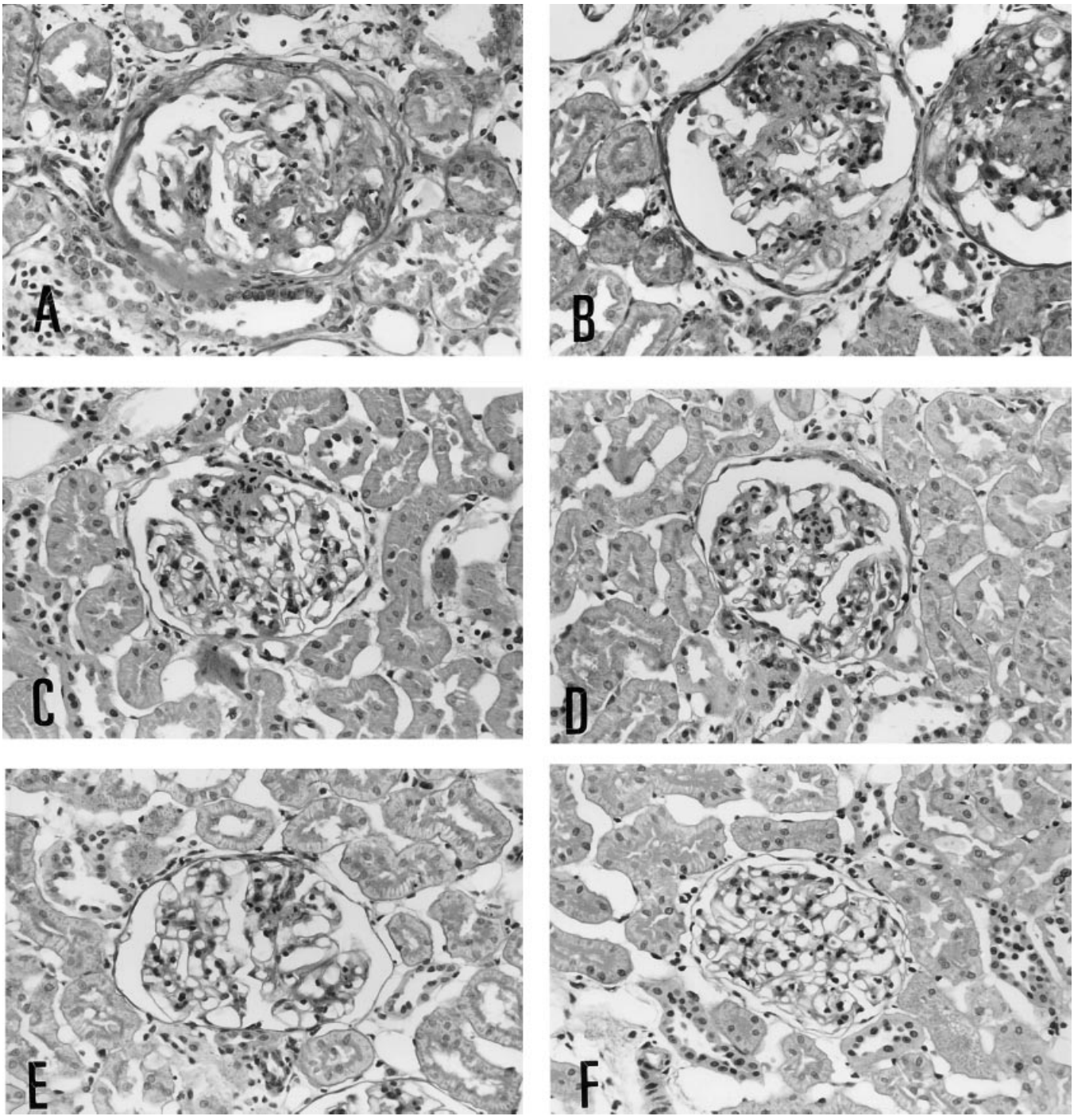

Fig. 4. Light micrographs of a glomerulus from untreated glomerulonephritis (GN) rats (*A*), hydralazine-treated GN rats (*B*), cilazapril-treated GN rats (*C*), cilazapril plus Hoe 140-treated GN rats (*D*), candesartan-treated GN rats (*E*), and nephrectomized control rats (*F*) (PAS stain final magnification ×560).

AT2R may contribute to the renoprotective effects of ACEIs and AT1R antagonists, respectively, to the same extent, we cannot conclude that the renoprotective effect of ACEIs is mediated by the reduction of Ang II generation, even if ACEIs and AT1R antagonists show the same beneficial effect. Thus, we compared an ACEI with an AT1R antagonist with respect to renoprotective effects and examined the effect of a B2 receptor antagonist on ACEI-induced renoprotection in rat mesangioproliferative GN.

Because we started the treatment on the same day that antibody was injected, it seems possible that Ang

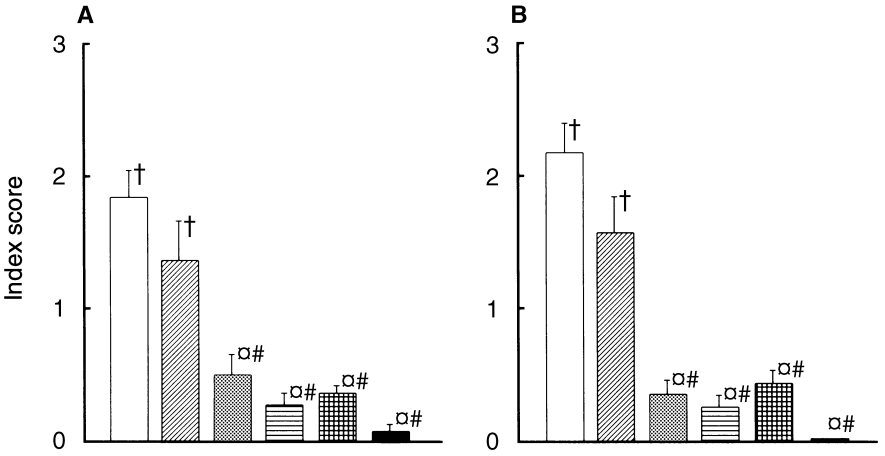

Fig. 5. Glomerulosclerosis index (GSI) and tubulointerstitial lesion index (TILI) in experimental nephritis. Symbols are: (□) untreated glomerulonephritis (GN) rats; (▨) hydralazine-treated GN rats; (▩) cilazapril-treated GN rats; (▤) cilazapril plus Hoe 140-treated GN rats; (▦) candesartan-treated GN rats; (■) nephrectomized control rats. $^{*}P < 0.001$ vs. untreated GN rats; $^{\square}P < 0.05$ vs. hydralazine-treated GN rats; $^{\dagger}P < 0.005$; $^{\#}P < 0.05$ vs. uninephrectomized control.

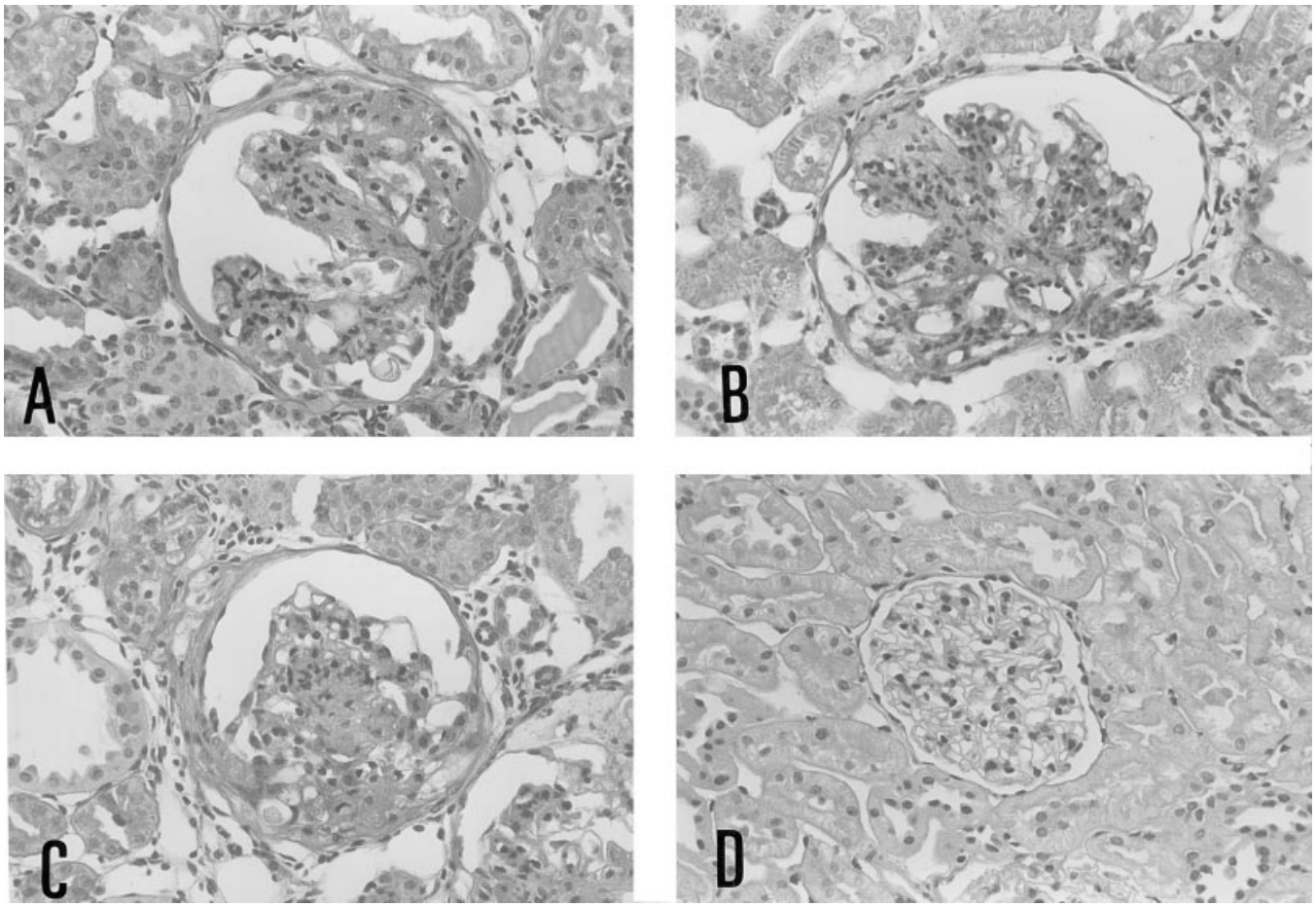

Fig. 6. Light micrographs of a glomerulus from untreated (*A*), daily intraperitoneally phosphate-buffered saline injected (*B*), subcutaneously minipump implanted (*C*) glomerulonephritis (GN) rats, and non-GN control rats (*D*) (PAS stain; final magnification, ×560).

II blockades ameliorate nephritis by changing the binding of the antibody in the kidney. To exclude this possibility, we examined the effect of antihypertensive agents on the binding of antibody in the kidney and found that antihypertensive agents did not change the binding of antibody in the kidney. We also found that antihypertensive agents did not ameliorate the increase in proteinuria one week after induction of the disease. These results indicate that antihypertensive agents did not affect the induction of the disease by changing the binding of anti-

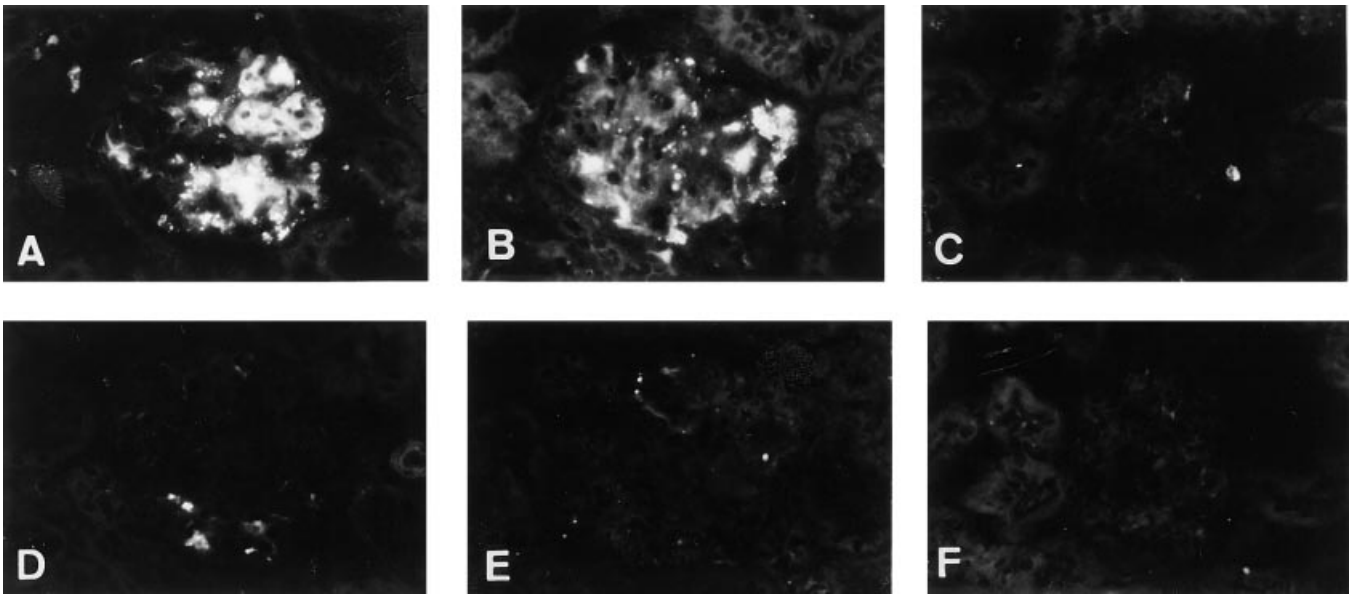

Fig. 7. Immunofluorescence micrographs of a glomerulus stained with anti-TGF-β antibody. (*A*) indicates untreated glomerulonephritis (GN) rats, (*B*) hydralazine-treated GN rats, (*C*) cilazapril-treated GN rats, (*D*) cilazapril plus Hoe 140-treated GN rats, (*E*) candesartan-treated GN rats, (*F*) nephrectomized control rats (final magnification ×350).

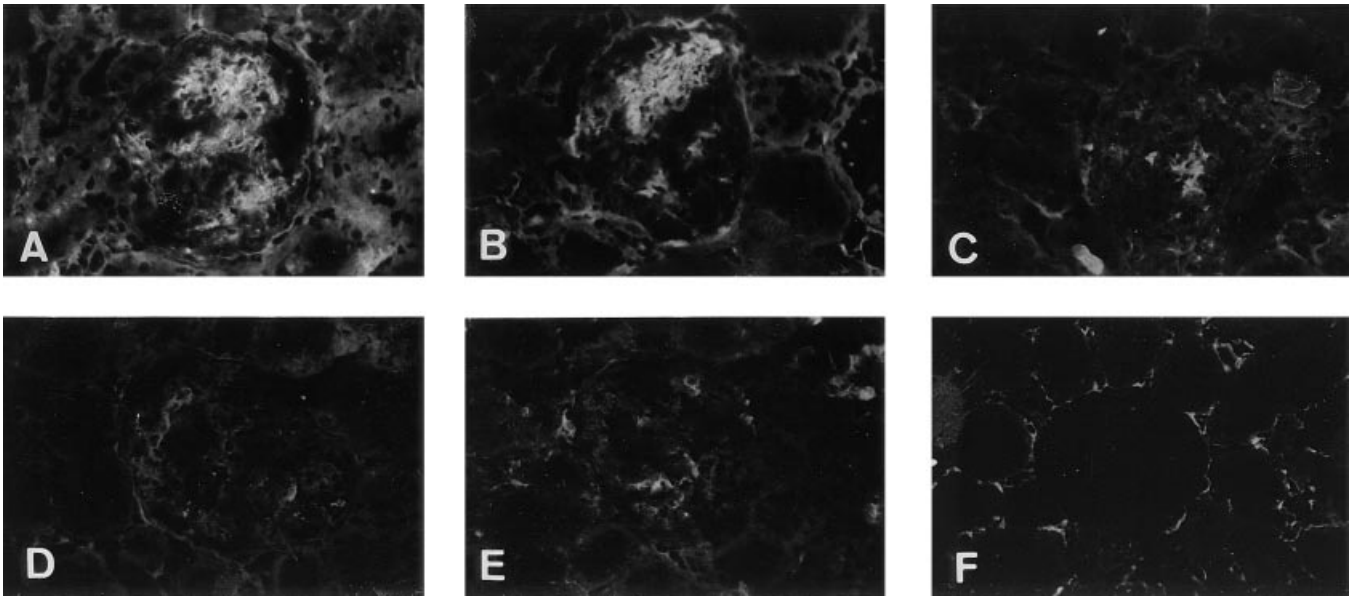

Fig. 8. Immunofluorescence micrographs of a glomerulus stained with antitype I collagen antibody. (*A*) indicates untreated glomerulonephritis (GN) rats, (*B*) hydralazine-treated GN rats, (*C*) cilazapril-treated GN rats, (*D*) cilazapril plus Hoe 140-treated GN rats, (*E*) candesartan-treated GN rats, (*F*) nephrectomized control rats (final magnification ×350).

body in the kidney. We also used daily intraperitoneal injections of candesartan and repeated subcutaneous minipump implantations to administer Hoe 140. To exclude the possibility that these manipulations could change the course of the disease, we examined the effects of manipulations and found that they did not affect the progression of the disease. There was no difference in body weight among the GN animal groups until six weeks after induction of the disease, suggesting that manipulations did not change the nutritional condition among the GN animal groups.

An *in vitro* study using isolated interlobular, efferent, and afferent arteriolar preparations demonstrated that bradykinin causes marked vasodilation only in the effer-

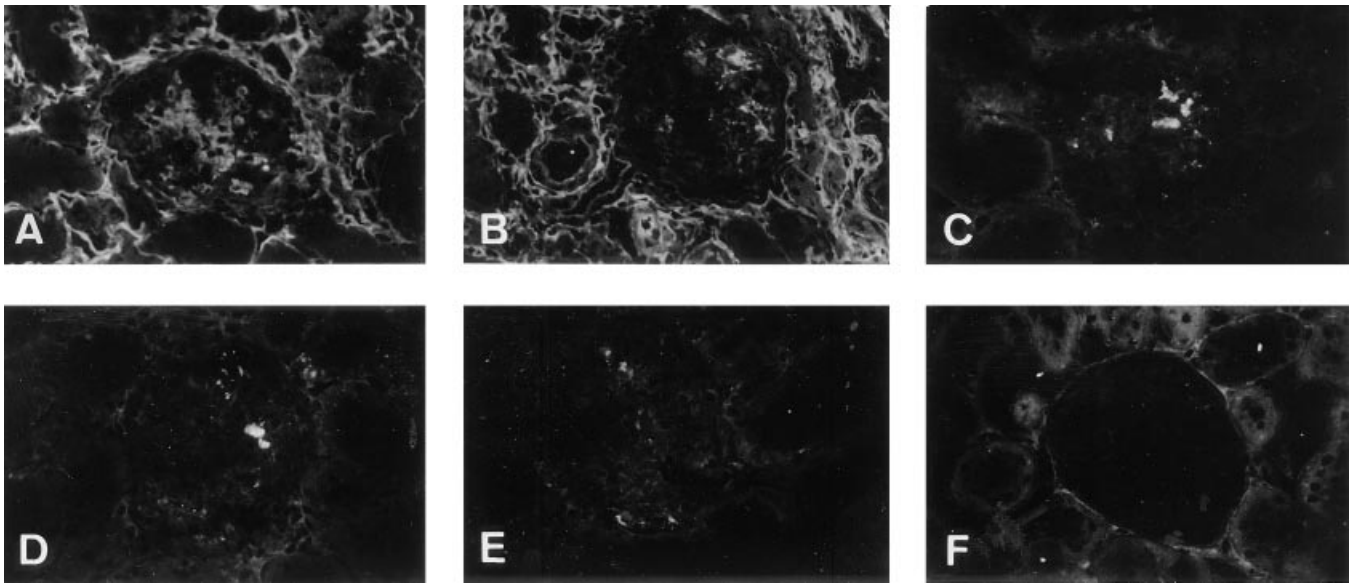

Fig. 9. Immunofluorescence micrographs of a glomerulus stained with antitype III collagen antibody. (*A*) indicates untreated glomerulonephritis (GN) rats, (*B*) hydralazine-treated GN rats, (*C*) cilazapril-treated GN rats, (*D*) cilazapril plus Hoe 140-treated GN rats, (*E*) candesartan-treated GN rats, (*F*) nephrectomized control rats (final magnification ×350).

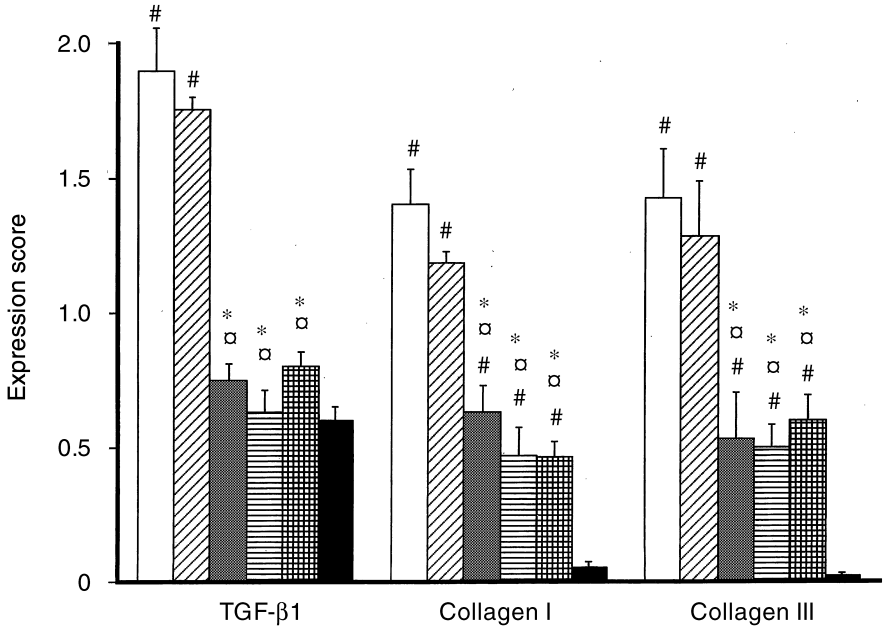

Fig. 10. Semiquantatative evaluation of immunohistochemistry in experimental glomerulonephritis. Symbols are: (□) untreated glomerulonephritis (GN) rats; (▨) hydralazine-treated GN rats; (▩) cilazapril-treated GN rats; (▤) cilazapril plus Hoe 140-treated GN rats; (▦) candesartan-treated GN rats; (■) nephrectomized control rats. $^{*}P < 0.01$ vs. untreated GN rats; $^{\square}P < 0.01$ vs. hydralazine-treated GN rats; $^{\#}P < 0.01$ vs. uninephrectomized control.

ent arteriole [9]. Both bradykinin type 1 (B1) and type 2 (B2) receptors have been identified in the glomerulus [50, 51]. The B1 receptor has a selective affinity for the natural metabolite des-Arg^9-bradykinin [52]. Bradykinin should predominantly be degraded into des-Arg^9-bradykinin under pathological conditions [51]. Collagen synthesis and cell proliferation are induced through the B1 receptor in rat mesangial cells [53]. The B1 receptor responses have been reported to be important in the generation and/or maintenance of renal vasoconstriction in disease states that lead to ESRF [54]. The B2 receptor is thought to mediate most of the physiological functions of bradykinin [9, 10], including vasodilation, diuresis, and formation of nitric oxide and prostacyclin. These findings suggest that if an ACEI-induced increase in bradykinin is involved in preventing the progression to ESRF, it should be mediated through the B2 receptor. Previous studies demonstrated that Hoe 140 is specific

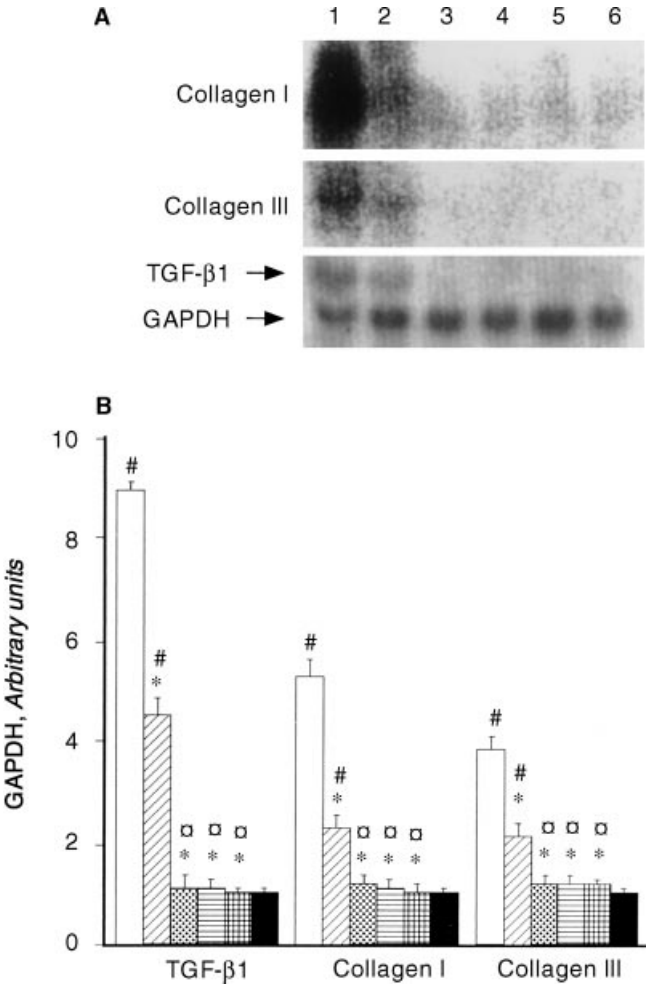

Fig. 11. (*A*) Northern blot analysis. Typical autoradiograms of mRNA for TGF-β (2.5 kb), collagen I (4.7 and 5.7 kb), collagen III (5.3 kb) from the renal cortex of experimental glomerulonephritis (GN) rats. (*B*) Renal cortical mRNA levels for TGF-β, collagen I (col. I), and collagen III (col. III). Symbols are: (□) untreated GN rats; (▨) hydralazine-treated GN rats; (▩) cilazapril-treated GN rats; (▤) cilazapril plus Hoe 140-treated GN rats; (▦) candesartan-treated GN rats; (■) nephrectomized control rats. $^{*}P < 0.01$ vs. untreated GN rats; $^{\square}P < 0.01$ vs. hydralazine-treated GN rats; $^{\#}P < 0.01$ vs. uninephrectomized control.

for kinin B2 receptors and does not influence the actions of other vasoactive hormones such as prostaglandins, norepinephrines, and Ang II [55–57]. Cilazapril and candesartan, but not hydralazine, reduced proteinuria and ameliorated the morphological lesions, although cilazapril, candesartan, and hydralazine reduced SBP to the same extent. Hoe 140 failed to blunt the cilazapril-induced renoprotection. These results suggest that most of the benefits conferred by cilazapril are achieved through the reduction of AT1R-mediated effects of Ang II and that neither kinin potentiation nor AT2R-mediated pathways contribute to the beneficial effect of RAS blockades in this model.

In the nephrotic phase of passive Heymann nephritis, an ACEI, but not an AT1R antagonist, reduced proteinuria [13], while aprotinin, a blocker of bradykinin production, blunted the antiproteinuric effect of ACE inhibition [12]. These results suggested that the antiproteinuric effect of ACEIs is mediated by an increase in bradykinin. Tanaka et al examined the mechanism by which an ACEI decreases proteinuria in puromycin aminonucleoside-induced nephrosis [47]. They found that ACEIs lessened proteinuria by increasing bradykinin in the early phase and by decreasing Ang II generation in the late phase. In addition, they demonstrated that enalapril and an AT1R antagonist improved the renal pathology, and they speculated that the subsequent progression of glomerulosclerosis is caused by the RAS-involving mechanism. This study demonstrated that proteinuria increases sharply one week after induction of the disease and then gradually increases until the end of the study. Cilazapril and candesartan did not reduce the early phase proteinuria, but did reduce the late-phase proteinuria. Hoe 140 did not abolish the ACEI-induced reduction of the late-phase proteinuria. The results suggest that the early-phase proteinuria is independent of Ang II and bradykinin, and that the late-phase proteinuria is due to the RAS-involving mechanism. This study also showed that, in contrast to hydralazine, RAS blockades produced the renoprotective effect, whereas RAS blockades and hydralazine failed to reduce the early phase proteinuria, suggesting that the renoprotective effect of a treatment cannot be predicted by whether the treatment reduces proteinuria in the early phase.

The participation of TGF-β in the progression of glomerulosclerosis was shown by the *in vivo* and *in vitro* studies of Border et al [20–22, 24, 58]. Ang II increases protein and mRNA levels of TGF-β and matrix components in cultured mesangial cells [24]. Our study showed that protein and mRNA levels of TGF-β and collagen types I and III increased in our model and that Ang II blockades, but not hydralazine, reduced these signals and ameliorated the disease. The results suggest that RAS blockades prevent the progression of the disease by reducing TGF-β synthesis. On the other hand, Shimizu et al found that pirfenidone decreased mRNA levels of TGF-β and ameliorated renal pathology, but failed to reduce proteinuria in a rat remnant kidney model [59]. In addition, our previous study with progressive mesangioproliferative GN showed that pirfenidone significantly reduced proteinuria, but not to the control levels [29]. ACEIs produce greater vasodilation in efferent arterioles, and these hemodynamic changes reduce the intraglomerular pressure, resulting in the reduction of proteinuria and in renoprotection [60]. Cyclic stretching of cultured mesangial cells that may occur in response to glomerular hypertension leads to increased synthesis of matrix proteins and TGF-β [61, 62]. Taken together, it seems likely that RAS blockades prevent the progression of the dis-

ease not only by reducing the production of TGF-β and matrix components, but also by modulating intrarenal hemodynamics.

In summary, we have demonstrated that (*a*) in progressive mesangioproliferative GN, an ACEI and an AT1R antagonist, but not hydralazine, attenuate the development of massive proteinuria, ameliorate morphological lesions, and reduce the protein and mRNA levels of TGF-β1 and collagens type I and III, and that (*b*) Hoe 140 did not blunt the ACEI-induced renoprotection. These findings suggest that ACEIs prevent the progression to ESRF by modulating the effects of Ang II via AT1R on the production of TGF-β and matrix components as well as on intrarenal hemodynamics in this rat model of progressive GN.

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Reprint requests to Takamichi Nakamura, M.D., Ph.D., Division of Blood Transfusion, Yamanashi Medical University, 1110 Shimokato Tamaho Nakakoma, Yamanashi 409-3898, Japan.
E-mail: nakamura@res.yamanashi-med.ac.jp

APPENDIX

Abbreviations used in this article are: ACEIs, angiotensin-converting enzyme inhibitors; Ang II, angiotensin II; AT1R, Ang II type 1 receptor; B1, bradykinin type 1; B2, bradykinin type 2; ESRF, end-stage renal failure; IF, Immunofluorescence; GN, glomerulonephritis; GSI, glomerulosclerosis index; PBS, phosphate-buffered saline; RAS, renin-angiotensin system; SBP, systolic blood pressure; TGF-β, transforming growth factor-β; TILI, tubulointerstitial lesion index; U_{Prot}, urinary protein excretion.

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